Characterization of Phytoconstituents, *In vitro* Antioxidant Activity and Pharmacological Investigation of the Root Extract of *Typhonium trilobatum*

Dissertation submitted to the Department of Mathematics and Natural Sciences, BRAC University in the partial fulfillment of the requirements for the Degree of Master of Science in Biotechnology

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Declaration

This is declared that the research work entitled "Characterization of phytoconstituents, *in vitro* antioxidant activity and pharmacological investigation of the root extract of *Typhonium trilobatum*" submitted by Fahim Shafa, has been carried out under the supervision of Dr. M. Mahboob Hossain, Associate professor, Microbiology programe, Department of Mathematics and Natural Sciences, BRAC University and co-supervision of Mr. Mohammad Shahriar, Assistant professor, University of Asia Pacific.

It is also declared that, the research has been done as the partial requirements of the M.S. degree in Biotechnology under the Department of Mathematics and Natural Sciences in BRAC University, Dhaka. It is further declared that the research work presented here is original and has not been submitted anywhere else for any degree or diploma.

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Abstract

In the present study, the phytochemical screening, *in vitro* antioxidant activity, thrombolytic activity, membrane stabilizing activity, acute toxicity and anti-depressant activity were evaluated using Forced Swimming Test (FST) of the root extract of *Typhonium trilobatum*. Phytochemical evaluation was done by performing different chemical tests and the presence of flavonoid, carbohydrate and phenol were detected in the root parts of the plant. Methanol, chloroform and ethanol were used as solvent in extraction process. Each extraction yield was analyzed using High Performance Liquid Chromatography (HPLC) to identify and measure the quantity of ascorbic acid. Total flavonoid content and total phenol content was also determined by UV–VIS Spectrophotometer. Ascorbic acid, flavonoid, phenol was found to be higher concentration in ethanol extract compare with methanol and chloroform extract. Ethanol was suitable solvent in extraction process of *Typhonium trilobatum*. *In vitro* antioxidant activity of the root extracts was performed by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging, cupric reducing antioxidant activity, scavenging of hydrogen peroxide and nitric oxide radical scavenging assay. These four methods were used to evaluate antioxidant activity and the root extract showed good potential. The root extract showed lower thrombolytic activity. Anti-depressant test was done by using forced swim test showed low dose anti-depressant activity. Our investigation indicates that *Typhonium trilobatum* roots contain high amount of ascorbic acid, phenolic and flavonoid compound which may responsible for its biological activities in folkloric medicine.

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Abbreviations

CUPRAC	Cupric reducing antioxidant capacity
DHEA	Dihydro eplandrosterone
DPPH	2,2-diphenyl-1-picrylhydrazyl
FST	Forced swimming test
GAE	Gallic acid equivalence
HCl	Hydrochloric acid
HOCl	Hypochlorous acid
HPLC	High performance liquid chromatography
ICDDR,B	International centre for diarrhoeal disease research, Bangladesh
MDA	Malondialdehyde
NO	Nitric oxide
PE	Pulmonary embolism
RBC	Red blood cell
ROS	Reactive oxygen species
SD	Standard deviation
SK	Streptokinase
TFC	Total flavonoid content
TPC	Total phenolic content

Chapter 1: Introduction

1.1 Background

Plants have been an important source of medicine for thousands of years. Even today, the World Health Organization estimates that up to 80 percent of people still rely mainly on traditional remedies such as herbs for their medicines. Plants are also the sources of many modern medicines. It is estimated that approximately one quarter of prescribed drugs contain plant extracts or active ingredients obtained from or modeled on plant substances. Herbs are turning to be a replacement for synthetics. The herbal products today are considered to be safer to human and environment. The practice of herbal medicine is wide spread in China, India, Japan, Pakistan, Srilanka, Thailand and Bangladesh. In China about 40% of the total medicinal consumption is attributed to traditional medicines.

Plants are good sources of natural antioxidants which provide protection against harmful free radicals and have been strongly associated with reduced risk of chronic diseases. Actually, free radical is naturally produced in our body by the metabolism of amino acids and fats. Oxidation is essential to many living organisms for the production of energy to fuel biological process. However the uncontrolled production of oxygen derived free radicals is involved in the onset of many diseases such as cancer, rheumatoid, arthritis and arteriosclerosis as well degenerative processes associated with aging (Halliwel, 1990). These free radicals are unstable and it can react with cells and destroy it. The bind of free radical with DNA structure will lead to mutation and it is cause of cancer. Free radicals formed in our body in several of type such as superoxide, hydroxyl and peroxyl. The antioxidant is needed to inhibit all the free radical from react. They scavenge radicals by inhibiting initiation and breaking of chain reaction, suppressing formation of free radicals by binding to the metal ions, reducing hydrogen peroxide and quenching superoxide and single oxygen (Shi *et al.,* 1991).

High concentrations of phytochemical in plant extracts are associated with strong antioxidant activity. Ascorbic acid and phenolic compounds including vitamins, pigments and flavonoids have been identified to be responsible for antioxidant properties in most plant. Herbs are turning to be a replacement for synthetics. The herbal products today are considered to be safer to human and environment. *Typhonium trilobatum* have the ability to synthesize a wide variety of chemical compounds that are used to perform important biological functions.

Typhonium trilobatum is widely used in folk medicine for the treatment of various diseases. The herb possess anti-diarrheal, antiseptic and analgesic qualities (Ali *et al.*, 2012). Thus, the present study evaluated the *in vitro* antioxidant potential of root extract of *Typhonium trilobatum.* The different antioxidant assays, including DPPH free radical scavenging, nitric oxide scavenging, hydrogen peroxide scavenging, reducing power and RBC membrane stabilization activity, were studied. Moreover, High Performance Liquid Chromatography (HPLC) was used to identify and quantify the ascorbic acid in the root extracts.

1.2 Objectives and Scopes of the Study

The objectives and the scopes of works are identified as follows:

- Phytochemical investigation by performing different chemical tests.
- Analysis of the extraction yield using High Performance Liquid Chromatography (HPLC) to identify and measure the quantity of ascorbic acid.
- Estimation of total flavonoid content and total phenol in the sample.
- Investigation of the antioxidant property of root extract of *Typhonium trilobatum* using various *in vitro* methods.
- Investigation of *in vitro* thrombolytic and membrane stabilizing activities.
- Investigation of anti-depressant activity by using forced swim test.
- Investigation of the acute toxicity using *in vivo* method.

1.3 Limitations of the Research

- Factors affecting oxidation reactions and antioxidant activities *in vivo* and *in vitro* differ. The current approaches have still left many open questions. *In vitro* assays can only rank antioxidant activity for their particular reaction system and their relevance to *in vivo* health protective activities is uncertain. At this time there is no convenient assay method to evaluate the antioxidant capacity *in vivo* the reaction can not be easily monitored in real time.
- Solvent selection for extraction procedure that can affect the extraction yield.
- The temperature during drying process might cause the degradation of some of the polyphenol substituents. phenol, flavonoids and ascorbic acid are very unstable compounds doing storage condition these compounds may be degraded.

1.4 Rationales and Significance

Typhonium trilobatum has a huge potential as an alternative source of antioxidant since the price is cheaper and has high antioxidant contents compared to traditional source spices and herbs. Therefore, root extract of *Typhonium trilobatum* can be a very important contributor in new drug discovery including antioxidant, antidepressant drug.

Chapter 2: Samples and Materials

2.1 Introduction

The mankind has been a victim of diseases since the very beginning of their existence. Whereas the nature provides remedies of every disease. Some natural plants are being used extensively as food and health supplements, which help to combat diseases. But medicinal plants may exhibit unwanted side effects due to the presence of some additional toxic constituents when used in crude form (Ghani, 2003). So phytochemical and pharmacological works have to be done to isolate the active constituents in the pure form to avoid adverse effects and to ensure safe use of herbal drugs. The isolated compounds may then use to develop new drugs in convenient dosage forms and this type of drug discovery is amazing. Almost 15% of the plants are known to have been investigated pharmacologically out of the estimated 5,00,000 species of higher plants growing on earth (Farnsworth, 1976).

Bangladesh has a rich and prestigious heritage of herbal medicine among the South Asian countries because of its suitable tropical climate and fertile soil. More than 500 species of medicinal plants are estimated as growing in Bangladesh and about 250 species of them are used for the preparation of traditional medicines. Traditional records and ecological diversity indicate that Bangladeshi plants (Table 2.1) represent an exciting resource for possible lead structures in drug design.

Table 2.1: Some medicinal plants of Bangladesh and their therapeutic uses

Scientific Name	Local name (Bengali)	Traditional uses
Adhatoda vesica	Basak	Cough, Asthma, Bronchitis, Pneumonia
Asparagus racemosus	Shatamuli	Urinary disorders, Jaundice
Azardirchata indica	Neem	Inflammation, Small pox
Centella asiatica	Thankuni	Skin problem, Digestive disorders, Leprosy, Dysentery
Rauwolfia serpentine	Sarpagandha	Hypertention, Insomnia, Anxiety, Insanity

Source: Ghani, 2003

2.2 The Studied Plant: *Typhonium trilobatum (L.) Schott*

Typhonium (Figure 2.1) is a genus in the *Araceae* family endemic to tropical Asia, the South Pacific, and Australia. It consists of approximately 50 species that are typically found growing in wooded area. Seven species of *Typhonium* grow in Bangladesh which are:

- *Typhonium blumei*
- *Typhonium flagelliforme*
- *Typhonium roxburghii*
- *Typhonium trilobatum*
- *Typhonium neogracile*
- *Typhonium listeri*
- *Typhonium cochleare*

Figure 2.1: *Typhonium trilobatum (L.) Schott*

Among the 7 (seven) species of *Typhonium* available in Bangladesh, *Typhonium trilobatum* (L.) Scott was selected for the current study. It is a neglected species of *Typhonium* genus on which very few scientific investigations have been conducted although it is widely used as traditional medicine. There remains a possibility that the plant may contain some bioactive compounds essential to treat diseases and so this plant is considered under the current phytochemical and pharmacological studies.

2.3 Taxonomic Hierarchy of *Typhonium trilobatum*

Kingdom: Plantae

Phylum: Magnoliophyta

Class: Liliopsida

Order: Arales

Family: Araceae

Genus: Typhonium

Species: *Typhonium trilobatum*

2.4 General Information of Plant

Typhonium trilobatum is a small tuberous herb, with *subglobose* tuber up to 4 cm diam. Petiole 25-30 cm long; lamina *hastate-subtrisect*, segments all acuminate, front segment ovate, 8-18 cm long, lateral ones obliquely ovate, shorter, *subbilobed* at base. Peduncle thin, 5-7 cm long; tube of spathe oblong, 2.5 cm long, lamina *oblong-ovate-lanceolate*, acuminate, 15 or more cm long, 5-7 cm broad, inside rose-purple. Spadix nearly 15 cm long. Female inflorescence short-cylindrical, about 7 mm long; male inflorescence 1.25-1.5 cm long, rose-pink, situated above the female. Flowering and fruiting time: April- October (Ghani, 2003).

2.5 Plant Names

Botanical Name: *Typhonium trilobatum (L.) Schott* (Figure 2.2).

Common Name: Bengal Arum.

Bengali Names: Ghet Kachu, Gher Kachu, Ghatkol, Kharkon, Khar Kachu, Cham Ghas.

Tribal Names: Kharbas, Nirbish, Sarakao (Chakma), Kalman (Garo).

Figure 2.2: *Typhonium trilobatum (L.) Schott*

2.6 Plant Distribution

Typhonium trilobatum is widely distributed in tropical and subtropical area around the world. It is found from Nepal to Southeast China, North Malaysia and Sri Lanka. It is also introduced in Philippines, West Borneo, Singapore, and West Africa. In Bangladesh, the plant is distributed throughout the country, but it is mainly found in Chittagong, Chittagong Hill Tracts, Tangail, Sylhet and Dhaka (Ghani, 2003).

2.7 Traditional Uses

The plant is hypnotic. Fresh corms are very acrid and a powerful stimulant; employed as a poultice in tumours. The corms are reported to relax the bowels and provide relief in haemorrhoids and piles. They are eaten with bananas to cure the stomach complaints. The Garo of Madhupur applies root paste locally on ulcer of cattle.

Chapter 3: Methodology

3.1 Introduction

Ordered and pre-determined steps have been selected from different literature reviews and followed accordingly to conduct the study. Following flowchart (Figure 3.1) summarizes the steps of the methodology.

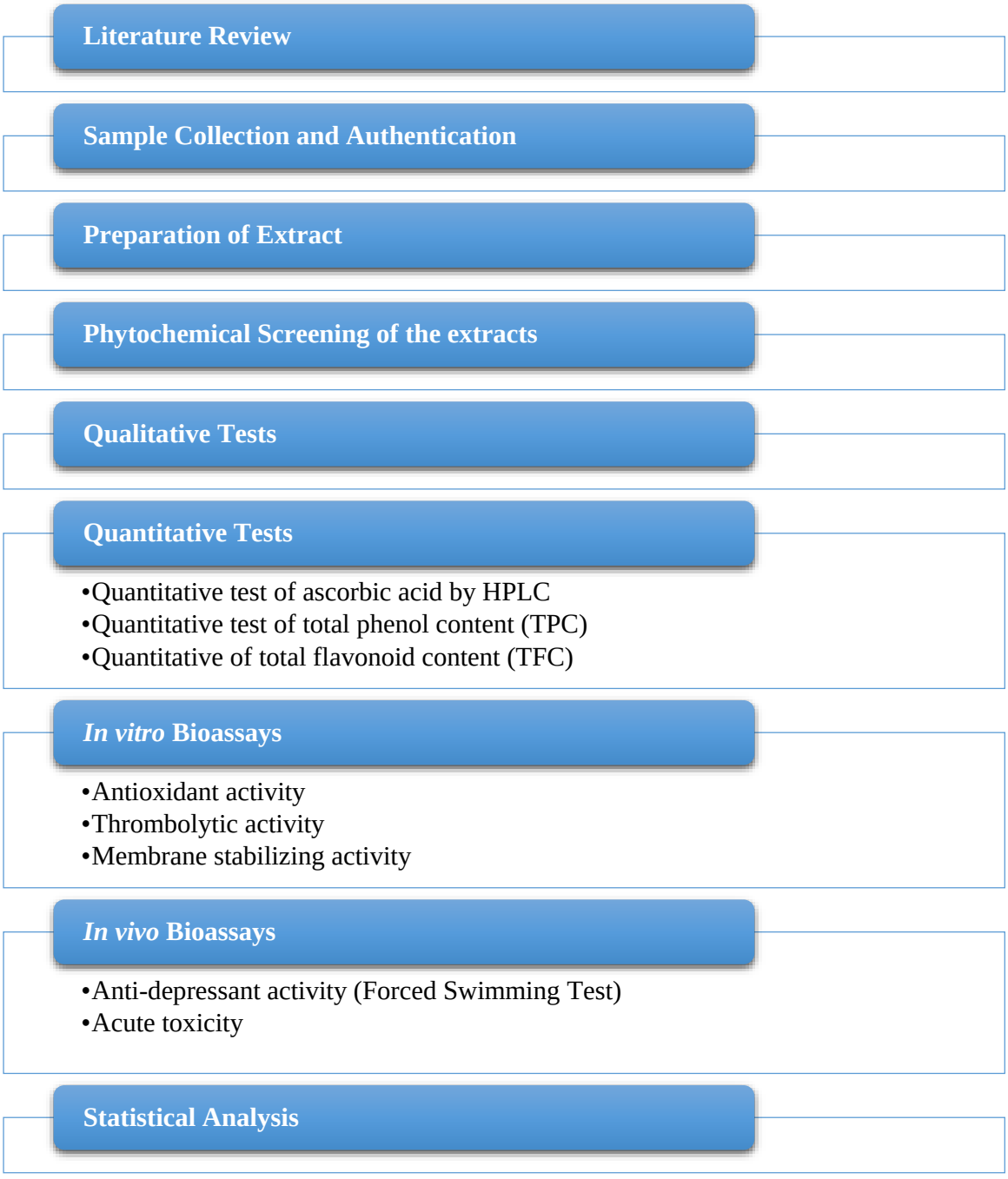

Figure 3.1: Methodology of the research

3.2 Literature Review

The ethanolic leaves extract of *Typhonium trilobatum* was investigated specifically for anti-inflammatory, analgesic and anti-diarrheal activity. The plant extract demonstrated a significant inhibition of writhing ($P<0.01$) compared with the control group in acetic acid-induced writhing test on mice. The extract also expressively inhibited the xylene induced ear edema formation ($P<0.05$). In anti-diarrheal test, the extract significantly decreased the frequency of defecation and increased the mean latent period ($P<0.01$) in castor oil-induced diarrheal model mice at the doses of 250 and 500 mg/kg body weight (Ali *et al.*, 2012).

In the experiment, *in vitro* somatic embryogenesis of *Typhonium trilobatum* was evaluated. In the study *in vitro tuberization*, embryo maturation and germination were achieved (Das *et al.*, 1999).

The target specific *larvicidal* potential of roots extract of *Typhonium trilobatum* beside mosquito *Culex quinquefasciatus* was investigated. In the study, 100% mortality of 1st instar mosquito larvae was recorded at 0.4% concentration after 72h of exposure of crude extract. 0.5% concentration produced 100% at 72h, 89.99% and 79.99% mortality of 2nd, 3rd and 4th instar larvae. 50 ppm methanol extract showed 73.67% mortality of 3rd instar mosquito larvae at 72h. 400 ppm concentration was responsible for 100% mortality in 24 h (Haldar *et al.*, 2011).

The potential wound healing activity of *Typhonium trilobatum* was studied and methanolic and ethyl acetate extract were found greater wound healing activity than chloroform extract (Roy *et al.*, 2012).

The antibacterial activity of ethanolic, methanolic, hexane, benzene, petroleum, ether and chloroform extracts of tuber of *Typhonium trilobatum (L.) Schott*, (family: *Araceae*) was evaluated. The extracts were tested against two gram-positive bacteria and eight gram-negative bacteria respectively by agar diffusion method. In the result, the Petroleum ether and Hexane extracts did not show any antibacterial activity. Ethanolic, methanolic extracts were found highly active and chloroform extract showed the moderate antibacterial activity whereas the benzene extract showed least antibacterial activity against the bacterial pathogens (Kandhasamy, 2008).

Another study was carried out to isolate, identify and partially purify lectin from rhizome of *Typhonium trilobatum*. The crude protein extract was prepared from the plant rhizome as the process of lectin isolation in the test. *Hemaglutination* activity assay and its agglutinated in human blood cells were used to identify the isolated protein. In the experiment, lectin was

purified by using DEAE-Cellulose column chromatography and UV Spectrophotometric methods. According to the experiment, it is evident that a soluble extract from the rhizome of *Typhonium trilobatum* has the *hemaglutination* action against human blood (O +ve) cell. This result showed that the rhizome contains lectin. *In vitro* study of the extract indicated antibacterial activity against *E.coli*. This result supported that medicinal can be used for diseases such as piles, skin disease or diarrhea. Brine-Shrimp bioassay has also showed the cytotoxic activity of the extract. According to the experiment result, the experimented plant may have anti-neoplastic activity (Biswas, 2014).

3.3 Sample Collection and Authentication

The sample plant ***Typhonium trilobatum (L.) Schott*** was collected from the premises of University of Dhaka in July, 2014. Then it was verified (Accession No.: 39647) by the National Herbarium of Bangladesh, Mirpur, Dhaka. It took almost 1 week to be identified and authenticated by taxonomist of the National Herbarium of Bangladesh.

Chemicals and Reagents: The source of all the chemicals used in the study were of analytical graded and the producer is Merck India Pvt. Ltd., India.

3.4 Preparation of the Extract

The collected plants were cleaned and the roots of the plants were separated. Then the roots were dried in the Sun for one week (up to dried out). It was then dried in oven for 60 hours at considerably low temperature ($< 40^0$C) for fine grinding. The dried roots were then crushed into coarse powder using high capacity grinding mill in the Phytochemical Research Laboratory, Department of Pharmacy, University of Asia Pacific, Dhaka, Bangladesh. The sample were then stored in air-tight container with proper markings for easy identification. The sample was kept in cool, dark and dry place for the later investigation.

Hot solvent extraction process was used while extracting of the plant material using soxhlet apparatus at elevated temperature. The temperature was controlled between 40^0C to 60^0C. methanol, chloroform and ethanol were used as solvents. The mentioned three solvents (400 ml of each) and 20 gm of each powder were used separately for the above mentioned purpose. A rotary evaporator has been used to evaporate the liquid and to obtain liquid plant materials. The thick materials were then kept in petridishes and allowed to become highest possible gummy concentrate. The crude extracts found in this way were then stored with proper labeling

in cold and dry place. All the extracts were kept in refrigerator 4^0C for future investigation after extraction. The yield of different extracts were calculated according to the following equation.

$$Extract\ yield\ \% = \frac{W_1}{W_2} \times 100$$

Here,

W_1= net weight of extract in grams obtained after extraction

W_2= total weight of powder in grams taken initially for extraction.

Following (Table 3.1) result was found following the above mentioned procedure.

Table 3.1: Different fractions of extracts of *Typhonium trilobatum*

Plant	Solvent	Yield (%)
Typhonium trilobatum	Methanol	22.05%
	Ethanol	24.2%
	Chloroform	10%

3.5 Qualitative Test of Phytochemicals Present in Roots of *Typhonium trilobatum*

Phytochemical screening was carried out to assess the qualitative chemical composition of roots extracts using standard qualitative chemical tests that employed precipitation and coloration procedures. These tests were carried out to identify the major natural chemical groups such as alkaloids, carbohydrate, flavonoids, glycosides, saponins, phytosterols and steroids, tannin, phenols (Tiwari *et al.,* 2011). Phytochemical examinations were carried out for all the extract as per the standard methods.

- **Detection of carbohydrate:** 0.5 gm of each extract were dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrate by following process:

  A) Molisch's test: 2 ml of each filtrate were treated with 2 drops of Molisch's reagent (alcoholic α-naphthol solution) and 2 ml concentration H_2SO_4 with the body of test tube. Formation of the violet ring at the indicates the presence of carbohydrates.
  B) Fehling's test: 2 ml of each filtrate were treated with Fehling's A & B solutions (ratio 1:1) and boiled for few minutes. Formation of brick color or red precipitate indicates the presence of reducing sugars.

- **Detection of tanin (Lead acetate test):** 0.5 gm extracts was taken in different test tubes. Then 5 ml distilled water were added on theses then mixed properly. Few drops

of lead acetate were added on it. Formation of fellow precipitate indicates presence of tannins.

- **Detection of phenols (Ferric chloride test):** Extracts was treated with 3-4 drops of ferric chloride (w/v) solution. Formation of bluish black color indicates the presence of phenols.
- **Detection of flavonoids:** Extracts were treated with a few drops of concentrated HCl 37%. Formation of immediate yellow color indicates the presence of flavonoids.
- **Detection of glycosides:** A small amount of an alcoholic extract of the plant material was taken in a test tube and dissolved in 1 ml of distilled water. Then few drops of aqueous sodium hydroxide solution (0.1N) were added. Development of yellow color indicates the presence of glycosides.
- **Detection of saponins (Froth test):** 0.1 gm powdered material was diluted with 10 ml water and boiled for 3-5 minutes. After cooling 5 ml transferred in another test tube and again diluted with 5 ml distilled water. This was shaken vigorously for 15 minutes. Formation of 1 cm layer or foam indicates the presence of saponins.
- **Detection of phytosterols/steroids (Libermann burchard's test):** Small amount of Petroleum ether extract was treated with 1 ml of chloroform and filtered. The filtrates were treated with 2 ml of acetic anhydride. Then 1 ml concentrated sulphuric acid is added. Formation of green color which blue on standing indicates the presence of phytosterols or steroids.
- **Detection of tannin (Lead acetate test):** 0.5gm of extract was taken in different test tubes. Then 5 ml distilled water were added on these then mixed properly. Few drops of lead acetate were added on it. Formation of fellow precipitation indicates presence of tannins.
- **Detection of phenols (Ferric chloride test):** Extracts were treated with 3-4 drops of 15% ferric chloride (w/v) solution. Formation of bluish black color indicates the presence of phenols.

3.6 Quantitative Test of Phytochemicals Present in Roots of *Typhonium trilobatum*

3.6.1 Quantitative Test of Ascorbic Acid by HPLC

The quantitative estimation of *Typhonium trilobatum* in roots according to the standard protocols by HPLC analysis.

3.6.1.1 Chromatographic Condition of HPLC Analysis:

Separation for qualitative and quantitative analysis of the ascorbic acid was performed by HPLC with a an Agilent 1100 Series HPLC (Agilent, Waldbronn, Germany) with UV detector (operated at 280 nm) and injection valve with 20-µL sample loop. Compounds were separated on a 4.6 mm × 250 mm, i.e., 5-µm pore size Xterra-C18 column protected by a guard column containing the same packing. The flow rate was 1.00 ml/minutes. Data were integrated by Shimadzu class VP series software and results were obtained by comparison with standards, samples and solutions were filtered through 0.45-µm Nylon filters (Millipore) before analysis by HPLC. Simple mobile phase was used as control for identification of blank peaks.

Diluent Preparation (1% Acetic Acid): 1 ml of acetic acid was dissolved in 100 ml distilled water.

Buffer Preparation: 0.8 gm Sodium pentanesulfonate was taken in 1000 ml volumetric flask and volume was adjusted with 1% acetic acid.

Mobile Phase Preparation: The mobile phase consisted of 80% Buffer solution and 20% HPLC grade methanol.

Standard Preparation: 10.52 mg of ascorbic acid was weighed accurately and transferred to 10 ml volumetric flasks. The standard was dissolved in 10 ml diluent to prepare standard stock solution of 1.1 mg/ml. Solutions were filtered through 0.45-µm Nylon filters (Millipore) before analysis by HPLC.

Sample Preparation: 2.26 mg of each extract which was equivalent of ascorbic acid was transferred to 10 ml volumetric flasks and added 3 ml diluent and sonicate by ultra sonicator water bath and diluted up to mark. The solution was transferred to a centrifuge tube and centrifuged at 2000 rpm for 30 min. After centrifugation the supernatant solution was filtered through Whatman number 41filter paper. The solution was filtered through a 0.45-µm membrane prior to injection into the HPLC.

Calculation of Assay of Ascorbic Acid:

$$\text{Assay of ascorbic acid} = \frac{\text{SW} \times 10 \times \text{PA} \times \text{SP}}{10 \times \text{Sample weight (mg)} \times \text{Standard peak area}}$$

Here, SW= standard weight (mg)

PA = sample peak area

SP = total weight of extract (mg)

3.6.2 Quantitative Test of Total Phenol Content (TPC)

Total phenolic content (TPC) in root extracts was determined described using the Folin-Ciocalteu Reagent (Singleton *et al.,* 1999*;* Velioglu *et al.,* 1998) by UV spectrophotometric. The extract and standard were diluted as 6.25 µg/ml to 200 µg/ml. Folin Ciocalteu Reagent (1:15 Folin Ciocalteu Reagent and Distilled Water) and 7.5% Sodium carbonate were added to each sample as well as to the standard. Then all samples and standard were incubated at 20^0C temperature for 1 hour. Gallic acid was used as standard for the experiment. Absorbance of different concentrated solution mixtures were measured at 765 nm using spectrophotometer against blank and the TPC in root extract in Gallic Acid Equivalence (GAE) was calculated using the following equation.

$$C = (c \times V)/m$$

Here, C= total content of phenolic compounds, mg/gm root extract, in GAE
c= the concentration of Gallic acid established from the calibration curve (mg/ml)
V = the volume of extract in ml
m = the weight of root extract in gm.

3.6.3 Quantitative Test of Total Flavonoid Content (TFC)

Total flavonoid content (TFC) was determined using the aluminum chloride colorimetric method as per the suggestion of Wang, 2000. Quercetin was used as standard. 5 ml Folin-ciocalteu reagent (previously diluted with water I:10 v/v) and 4 ml (7.5% sodium carbonate) of sodium carbonate were added to each test tube containing 1 ml of diluted solution of sample as well as to the standard. Then the samples were incubated at 20^0C temperature for 1 hour and the standard diluted solution-reagent mixture was incubated at 20^0C temperature for 30 minutes. Absorbance was measured at 415 nm using spectrophotometer against blank. The total content of flavonoid compounds in plant methanol extracts in Quercetin equivalents was calculated using the following equation:

$$C = (c \times V)/m$$

Here, C= total content of phenolic compounds, mg/gm root extract, in Quercetin equivalent,
c= the concentration of Quercetin established from the calibration curve (mg/ml),
V = the volume of extract in ml
m = the weight of root extract in gm.

3.7 *In vitro* Bioassays

3.7.1 Antioxidant Activity

The *In vitro* antioxidant activities were determined by using four established methods. They are:

i. DPPH free radical scavenging assay
ii. Cupric Reducing Antioxidant Capacity (CUPRAC)
iii. Nitric oxide radical scavenging assay
iv. Scavenging of hydrogen peroxide

For every antioxidant activity test, stock solution was prepared by dissolving extract into ethanol. The concentration of the solution was 5 µg/ml from where six consequent experimental concentrations were prepared by serial dilution (Table 3.2). Standard solution was prepared using the stock preparation procedure by replacing extract with corresponding standard solution. Control sample was prepared containing the same volume without any extract and standard.

Table 3.2**:** Serial dilution performed in antioxidant activity evaluation

Concentration (µg/ml)	**Solution taken from stock solution**	**Solution taken from previous test tube**	**Adjust the volume by absolute ethanol**	**Final volume**
200	80 µl	-	1920 µl	2 ml
100	-	1 ml (200 µg/ml)	1 ml	2 ml
50	-	1 ml (100 µg/ml)	1 ml	2 ml
25	-	1 ml (50 µg/ml)	1 ml	2 ml
12.5	-	1 ml (25 µg/ml)	1 ml	2 ml
6.25	-	1 ml (12.5 µg/ml)	1 ml	2 ml

3.7.1.1 DPPH Free Radical Scavenging Assay

DPPH is a common abbreviation for an organic chemical compound 2,2-diphenyl-1-picrylhydrazyl. It is a used to determine the free radical scavenging capacity of the extracts. It is a dark-colored crystalline powder contained stable free-radical molecules. DPPH has two major applications, both in laboratory research: one is a monitor of chemical reactions involving radicals, most notably it is a common antioxidant assay and another is a standard of the position and intensity of electron paramagnetic resonance signals.

1 ml of the roots extract was taken in test tube and freshly prepared 2 ml of 0.004% DPPH solution was added in the test tubes for the final volume of 3 ml. Then the mixture was incubated in room temperature for 30 minutes. The control sample was prepared having the same volume without any extract as well as standard. Absorbance of each concentration solution were taken at 517 nm and free radical scavenging activity of the extracts. Then it was evaluated as % inhibition and/or IC_{50} using the equation below:

$$\%\ inhibition = (1 - \frac{A_I}{A_o}) \times 100$$

Here, A_I = absorbance of the extract or standard

A_0 = absorbance of the control.

IC_{50} is the concentration at which 50% of the total DPPH free radical is scavenged or neutralized and determined by plotting % inhibition against corresponding concentration.

3.7.1.2 Cupric Reducing Antioxidant Capacity (CUPRAC)

Antioxidants are health beneficial compounds through their combat with reactive oxygen and nitrogen species and free radicals that may cause tissue damage by leading to various diseases. It is evident that the development of a simple and widely applicable antioxidant capacity index for dietary polyphenols, vitamins C and E, and plasma antioxidants utilizing the copper(II)-neocuproine (Cu(II)-Nc) reagent as the chromogenic oxidizing agent. This novel method based on an electron-transfer mechanism was named by our research group as Cupric Reducing Antioxidant Capacity (CUPRAC) method.

0.5 ml of root extracts was taken in test tube and 1 ml of copper(II)chloride solution (0.01 M prepared from $CuCl_2.2H_20$), 1 ml of ammonium acetate buffer (at pH 7.0), 1 ml of neocaproin solution (0.0075 M) were added. 0.6 ml of distilled water was added to the final volume of the mixture and adjusted to 4.1 ml. The mixture was incubated for 1 hour at room temperature. The developed absorbance was measured at 450 nm.

3.7.1.3 Nitric Oxide Radical Scavenging Assay

Nitric oxide scavenging assay was performed according to the previously established method using sodium nitroprusside (Govindanrajan *et al.*, 2003). It was determined by the use of the Griess-Ilosva reaction. 2 ml of 10 mM sodium nitroprusside in 0.5 ml phosphate buffer saline (PH 7.4) was mixed with 0.5 ml of extract/sub-fraction of different diluted (6.25 µg/ml to 200

µg/ml) concentrations solution. The mixture was incubated at 25^0C for 150 minutes and 0.5 ml was taken out from the mixture and added into 1.0 ml sulphanilamide solution (0.33% in 20% glacial acetic acid) which was further incubated at room temperature for 5 minutes. Finally, 1.0 ml Naphthyl ethylenediamine dihydrochloride (0.1% w/v) was added and maintained at room temperature for 30 minutes. Sodium nitroprusside is known to decompose in aqueous solution at physiological pH (7.2) producing NO. Under aerobic conditions, NO reacts with oxygen to produce stable products (nitrate and nitrite), the quantities of which can be determined using Griess reagent (Marcocci *et al.,* 1994). The absorbance was measured at 546 nm and nitric oxide scavenging activity was evaluated as % inhibition and IC_{50} using the above equation used in DPPH free radical scavenging assay.

3.7.1.4 Scavenging of Hydrogen Peroxide

The ability of root extracts to scavenge hydrogen peroxide can be estimated according to the method of (Jayaparakasha, 2000). A solution of hydrogen peroxide (40 Mm) is prepared in phosphate buffer pH 7.4. The concentration of hydrogen peroxide is measured by absorption at 230 nm (0.1 mg/ml of the extract was added to hydrogen peroxide solution) using a spectrophotometer against a blank solution containing phosphate buffer without hydrogen peroxide. Hydrogen peroxide scavenging activity was evaluated as % inhibition and/or IC_{50} using the above equation used in DPPH free radical scavenging assay.

3.7.2 Thrombolytic Activity

3.7.2.1 Blood Sample

The blood was drawn from healthy human volunteers (n = 5) without a history of oral contraceptive and anticoagulant therapy. 500 µl of blood was transferred to previously weighted micro-centrifuge tubes and was allowed to form clots.

3.7.2.2 Streptokinase (SK)

Commercially available lyophilized Altepase (Streptokinase) vial (Trade name: S-Kinase from Popular Pharmaceuticals Ltd.) of 15,00,000 I.U. was collected. Then, 5 ml of 0.9% NaCl was added and mixed properly to prepare the concentration 3,00,000 I.U. This suspension was used as stock where 100 µl was used for *in vitro* thrombolysis.

The thrombolytic activity of all extracts were evaluated by using *in vitro* clot lysis method developed by Prasad *et al.,* 2006 and Ratnasooriya *et al.,* 2008 with slight modification, using

streptokinase (SK) as the standard substance. Human blood was allowed to clot. The different extracts of *Typhonium trilobatum* roots were added to the clotted blood. Thrombolytic activity was evaluated by determining % clot lysis using the following equation.

$$\% \text{ of clot lysis} = (W_2\text{-}W_3/W_2\text{-}W_1) \times 100$$

Here, W_1= weight of micro-centrifuge tube alone

W_2= weight of micro-centrifuge tube with blood clot

W_3= weight of micro-centrifuge tube after clot lysis

3.7.3 Membrane Stabilizing Activity

The erythrocyte membrane resembles to lysosomal membrane and as such, the effect of drugs on the stabilization of erythrocyte could be extrapolated to the stabilization of lysosomal membrane (Omale, 2008).

The membrane stabilizing activity of the extractives was assessed by using hypotonic solution-induced and heat-induced mice erythrocyte haemolysis (Shinde *et al.*, 1999).

To prepare the erythrocyte suspension, whole blood was obtained from healthy human volunteer and was taken in syringes (containing anticoagulant 3.1% Na-citrate). The blood was centrifuged and blood cells were washed three times with solution (154 mM NaCl) in 10 mM sodium phosphate buffer (pH 7.4) through centrifugation for 10 min at 3000 g.

3.7.3.1 Hypotonic Solution-induced Haemolysis

The test sample comprised of stock erythrocyte suspension (0.5 ml) and mixed with 5 ml hypotonic solution (50 mM NaCl) in sodium phosphate (10 mM) buffered saline (pH 7.4) containing the extract and acetyl salicylic acid (1.0 mg/ml). The control sample consisted 0.5 ml of RBCs mixed with hypotonic-buffered saline. The mixture was then incubated for 10 min (at room temperature), then centrifuged for 10 min at 3000 g. The absorbance of the supernatant was measured at 540 nm. The percentage inhibition of either haemolysis or membrane stabilization was calculated using the following equation:

$$\% \text{ inhibition of haemolysis} = 100 \text{ x } (OD_1\text{-}OD_2/OD_1)$$

Here, OD_1 = optical density of hypotonic-buffered saline solution alone (control)

OD_2 = optical density of test sample in hypotonic solution.

3.7.3.2 Heat- induced Haemolysis

Isotonic buffer containing aliquots (5 ml) of the different extracts were put into two duplicate sets of centrifuge tubes. The vehicle, in the same amount, was added to another tube as control. Erythrocyte suspension was added to each test tube and mixed properly. One pair of the tubes was incubated at 54^0C for 20 min in a water bath, while the other pair was maintained 0^0C to 5^0C in an ice bath. The reaction mixture was centrifuged for 3 min at 1500g and the absorbance of the supernatant was measured at 560 nm. The percentage inhibition or acceleration of haemolysis in tests and was calculated according to the equation:

$$\% \text{ Inhibition of haemolysis} = 100 \times [1- (OD_2-OD_1/ OD_3-OD_1)]$$

Where, OD_1 = optical density of unheated test sample

OD_2 = optical density of heated test sample

OD_3 = optical density of heated control sample.

3.8 Pharmacological Investigation

3.8.1 Collection and Preparation of Experimental Animals

Sixteen numbers of Swiss Albino mice of either sex were collected for the *in vivo* investigations from the animal resource department of ICDDR,B. The age of the mice were 6-7 weeks, weighing between 10-25 gm. Animals were maintained under standard environmental conditions (temp. 27 ± 1^0C, RH 55-65% and 12 light/12 hr dark cycle) and free access to feed and water was confirmed. The animals were acclimatized to laboratory environment for one week before the experiments performed. The mice were coded as I, II, III, IV and none for the number five on their tails.

3.8.2 Design of Experiment and Animal Grouping

The test animals were divided into four groups of two animals in each group (Table 3.3).

Table 3.3: Group distribution and administered substances with their doses

Group	Administered extract	Dose
1	Standard	Specific for each test
2	Methanol extract	100 mg/kg
3	Ethanol extract	100 mg/kg
4	Chloroform extract	100 mg/kg

3.8.3 *In vivo* Bioassays of Anti-depressant Activity (Forced Swimming Test, FST)

The Forced Swimming Test (FST) is the most widely used pharmacological *in vivo* model for assessing the antidepressant activity of any substance and was performed according to the method of Porsolt *et al.*,(1977).

A clear square water tank apparatus (30 cm x 20 cm x 20 cm) was filled with water (temp. 25 ± 1^0C) up to 9 cm. Mice were grouped as mentioned before (Table 3.3). Diazepam 2 mg/kg was used as reference drug and applied almost one hour before the final test. In the pre-test session, 24 hours before the test, every mice was placed individually into the cubical glass apparatus for 10 min. After oral dose administration, the test was performed by letting the mice to swim for 5 min each and their duration of immobility was recorded. The period when no further attempt to escape were made (apart from the necessary to keep its head above the water) was counted as immobile time.

3.8.4 Acute Toxicity due to the Experiment

Acute toxicity describes the adverse effects of a substance which results either from a single exposure or multiple exposures in a short period of time, usually less than 24 hours.

The test samples were applied orally to the test animals at different concentrations of 100 mg/kg (as per body weight). The animals were then observed for any mortality or any other sign of toxicity for every 6 hour for next 12 hours after applying the doses. Later, the animals were also kept under observation for 1 week (Walum, 1998).

3.9 Statistical Analysis of the Experiment Data

All data are presented as a mean ±Standard deviation (SD). IC_{50} values for scavenging of free radicals by the extracts were calculated from the dose - response curve by using Microsoft Excel-2010.

Chapter 4: Results and Discussion

4.1 Introduction

A number of different tests has been done under the experiment. The tests have been summarized in the following paragraphs.

4.2 Phytochemical Screening

Typhonium trilobatum is being used as the traditional medicine not only in different parts of Bangladesh but also throughout the world for the time immemorial. The result of the phytochemical screening of root of this plant directly correlates with the facts of using this plant as an ethnomedicine. The presence of flavonoids, carbohydrate and phenol which are essential constituents of the herbal medicines and also determined the quantity of some of the phytochemicals and phytonutrients. No glycoside, tannin, steroid and saponin were detected in this plant.

The root of *Typhonium trilobatum* showed either presence or absence of different phytochemicals. The results are listed below in the Table 4.1:

Table 4.1**:** Results of chemical group test of various root extracts of *Typhonium trilobatum*

Name of test	**Name of the root extract**		
	Methanol	**Ethanol**	**Chloroform**
Flavonoid	+	+	+
Carbohydrate	+	+	+
Glycoside	-	-	-
Tannin	-	-	-
Steroid	-	-	-
Phenol	+	+	+
Saponin	-	-	-

[+ = presence in bioactive compound, - = absence.]

4.3 Discussion of Quantification of Ascorbic acid by HPLC

The contents of the ascorbic acid in the root extract of *Typhonium trilobatum* were analyzed by HPLC. Based on the comparison of the retention times with the standard peak of ascorbic acid,

three extracts were identified, retention time was 2.5 minutes respectively. The most abundant ascorbic acid obtained from the ethanol extract was 51.13 mg/ 0.25 gm dry extract. Methanol extract also contained 48.16 mg whereas chloroform extract contained 44.65 mg of ascorbic acid per 0.25 gm total extract. According to this result we have to be said that root of *Typhonium trilobatum* has good antioxidant properties. The assay has been successfully used to quantify ascorbic acid in roots and being simple and independent of other antioxidant measurement commonly employed.

4.3.1 Quantitative Test of Total Phenol Content

Phenolic, ubiquitous to the plant kingdom are composed of several classes of compounds including flavonoids (flavones, isoflavones and flavonones), anthocyanins and catechins. They possess an ideal structure, chemistry for free radical scavenging activity. Antioxidant properties of polyphenol aries from their high reactivity as hydrogen or electron donors which can stabilized and delocalized the unpaired electron (chain–breaking function) and from their potential to chelate metal ions**.** Polyphenols are secondary metabolites of plants. Plant polyphenols offered some protection against the development of cancers, cardiovascular diseases, diabetes, osteoporosis and neurodegenerative diseases. Although the active components of the medicinal plant compounds are not known, polyphenols have received growing attention because of some exciting new findings concerning their biological activities. Pharmacologically, the antioxidant potential of polyphenolic compounds, particularly free radical scavenging and inhibition of lipid peroxidation are the most important. Polyphenol have the ability to block LDL oxidation and also decreases the formation of atherosclerotic plaques and decrease arterial stiffness, send-off arteries more responsive to endogenous stimuli of vasodilation (Moline *et al.,* 2000; Arai *et al.,* 2000; Duthie *et al.,* 2000). Furthermore, polyphenols play an important role in anticarcinogenic effects and inhibit the cytochrome P450 of enzymes that metabolizes many pro-carcinogens to reactive compounds, thus reducing the formation of reactive intermediates (Stoner, 1995). In addition, polyphenol have been shown to inhibit lipoxygenase and cyclogenase activity leading to lower aggregation of platelets and reduction of thrombotic tendency (Moline *et al.,* 2000). This ability is believed to be mainly due to their redox properties (Zheng *et al.,* 2001). The results strongly suggest that phenolics are important components of the tested plant extracts.

Total phenolic content of the different extracts of was determined by using the Folin-Ciocalteu reagent and were expressed as Gallic Acid Equivalents (GAE) per gram of plant extract. The

total phenolic contents of the test fractions were calculated using the standard curve of Gallic acid (y = 0.0027 x + 0.0832; R^2 = 0.9973) (Figure 4.1).

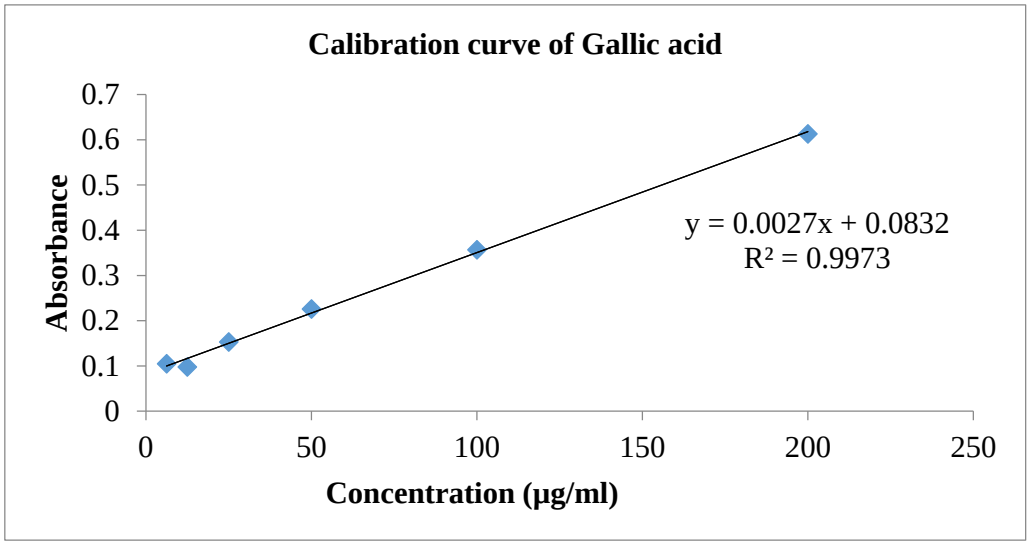

Figure 4.1: Calibration curve of Gallic acid

The highest concentration of phenols was measured in ethanolic extracts. Methanol and chloroform extracts contain considerably smaller concentrations of phenols. The total phenolic contents in plant extracts of the *Typhonium trilobatum* depend on the type of extract, i.e. the polarity of solvent used in extraction. The high solubility of phenols in polar solvents provides a high concentration of these compounds in the extracts obtained using polar solvents for the extraction. In the present investigation an ethanol extract of root of the plant was found to contain the highest amount of phenolic content (14.06 mg/gm) (Table 4.2). Phenolic contents of the extracts were found to decrease in the following order: Ethanol extract > Methanol extract > Chloroform extract.

Table 4.2: Total phenolic contents of different root extracts of *Typhonium trilobatum*

Extracts	Total phenolic content (mg/gm, Gallic acid equivalents)
Methanol extract	12.88±2.01
Ethanol extract	14.06±1.70
Chloroform extract	12.51±2.25

[Values are Mean ± Standard deviation]

4.3.2 Quantitative of Total Flavonoid Content (TFC)

Every group of flavonoids have their capacity to act as antioxidants depends upon their molecular structure. The position of hydroxyl groups in the chemical structure of flavonoids

are important for their antioxidant and free radical scavenging activities. Quercetin is a potent antioxidant because it has all the right structural features for free radical scavenging activity. Body cells and tissues are continuously threatened by the damage caused by free radicals and reactive oxygen species, which are produced during normal oxygen metabolism or are induced by exogenous damage (Grace *et al.,* 1994). This cellular damage causes a shift in the net charge of the cell, changing the osmotic pressure, leading to swelling and eventually cell death. Free radicals can attract various inflammatory mediators, contributing to a general inflammatory response and tissue damage. Naturally, there is a dynamic balance between the amount of ROS generated and degraded in cells. They are degraded to non-reactive forms by enzymatic and non-enzymatic antioxidant defenses produced in cells or by others supplied with the diet. Biological and pharmacological properties of flavonoids depends on their antioxidant activity (Birt *et al.,* 2001). Flavonoids have several different mechanisms of antioxidant properties, such as scavenging of free radicals, chelation of metal ions, such as iron and copper and inhibition of enzymes responsible for free radical generation (Benavente-Garcia *et al.,* 1997). These compounds in human diet can easily adjust antioxidants in the epithelial cell membrane walls against the attack of ROS. Depending on their structure, flavonoids are able to scavenge practically all known ROS.

The method of Aluminum chloride colorimetric was used to determine the total flavonoid contents of the different extracts of *Typhonium trilobatum.* Total flavonoid contents (TFC) was calculated using the standard curve of Quercetin ($y = 0.0048 x + 0.015$; $R^2 = 0.999$) (Figure 4.2) and was expressed as Quercetin Equivalents (QE) per gram of the root extract. Ethanol extract of *Typhonium trilobatum* leaves was found to contain the highest amount of flavonoids content (6.34 mg/gm).

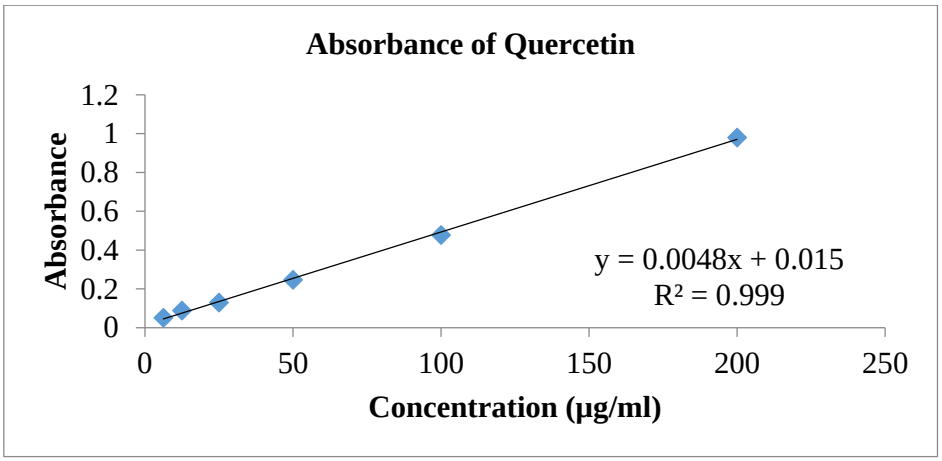

Figure 4.2: Calibration curve of Quercetin

Table 4.3 shows flavonoid contents of the extracts were found to decrease in the following order: Ethanol extract > Methanol extract > Chloroform extract. Therefore, the result suggested flavonoids may be the major contributors for the anti-oxidative properties and inhibitory actions toward the oxidative reaction.

Table 4.3: Total flavonoid contents of different root extracts of *Typhonium trilobatum*

Extracts	Total flavonoid content (mg/gm, Quercetin equivalents)
Methanol extract	5.06 ±0.98
Ethanol extract	6.34 ±0.96
Chloroform extract	4.88±0.99

[Values are Mean ± Standard deviation]

4.4 *In vitro* Bioassays

4.4.1 Antioxidant Activity

4.4.1.1 DPPH Free Radical Scavenging Assay

The antioxidant activity of all root extracts was estimated by DPPH assay. The DPPH assay is a very reliable and convenient method for screening antioxidant molecules. The reaction can analyze by simple spectrophotometric assay. DPPH free radical contains odd electron and shows maximum absorbance at 517 nm. When DPPH accept an electron donated by an antioxidant compound, its molar absorptivity reduces and the DPPH decolorized the color turn into purple to yellow, this visual color changed confirm the antioxidant properties of root extract. DPPH radical scavenging activity describes as IC_{50} values which is indicated concentration of the sample to produce a 50% reduction of free radicals.

Figure 4.3 showed the trend of the inhibition percentage of all sample extract including reference standard (ascorbic acid, methanol extract, ethanol extract, chloroform extract). The sequences of the inhibition percentages of all sample extract including reference standard are as follows Ascorbic acid> Ethanol extract> Methanol extract> Chloroform extract. The ethanol, methanol and chloroform extracts showed maximum activity of 87.39%, 84.39% and 83.52% inhibition at 200 µg/ml concentration, whereas standard ascorbic acid showed maximum 95.13% inhibition at the same concentration. Basically, the trend followed the polarity of solvent. Ascorbic acid had been reported to exhibit greater potential antioxidant activity (Padayatty *et al.,* 2003). The phenomenon in this study is acceptable since ascorbic acid has the highest inhibition percentage inhibits free radical activity.

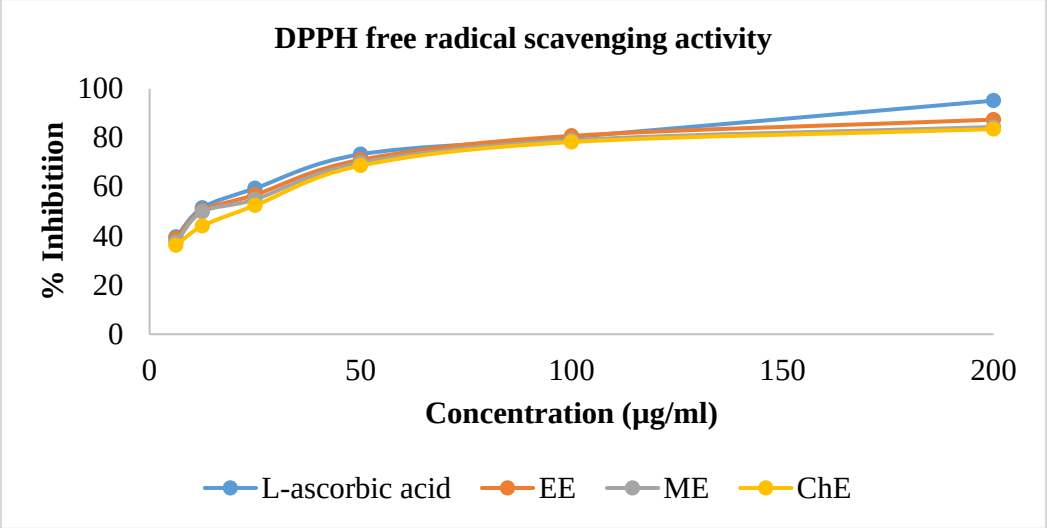

Figure 4.3**:** DPPH free radical scavenging activity of different root extracts of *Typhonium trilobatum*

The IC_{50} values of different root extracts of *Typhonium trilobatum* are presented in Table 4.4. IC_{50} of ascorbic acid was found 12.25 µg/ml. In comparison to standard, methanol, ethanol and chloroform extracts showed IC_{50} values of 14.32, 13.12 and 53.45 µg/ml respectively.

Table 4.4: IC_{50} values of different root extracts of *Typhonium trilobatum* in DPPH free radical scavenging assay

Sample	IC $_{50}$ value (µg/ml)
Methanol	14.32
Ethanol	13.12
Chloroform	53.45
Ascorbic acid	12.25

4.4.1.2 Cupric Reducing Antioxidant Capacity (CUPRAC)

The chromogenic oxidizing reagent of the development CUPRAC method that is (neocaproine) copper(II)chloride [Cu(II)-Nc] reacts with polyphenols [Ar(OH)n] in the manner:

$$2nCu(Nc)_2^{2+} + Ar(OH)_n = 2nCu(Nc)^{2+} + Ar(O)_n + 2nH^+$$

Where the liberated protons may be buffered with the relatively concentrated ammonium actate buffer solution. In this reaction assay of cupric reducing antioxidant capacity is based on the reduction of a Cu(II) complex the presence of antioxidants, in this case the color change is related to the reduced form of the complex which can be monitored at 450 nm wavelength. Absorbance indicates in reducing power capacity. The advantages of cupric assay are the working pH, which is closer to the physiological conditions pH 7.

In general the copper based assays target the thiol group containing antioxidant species. The sample which is reducing power capacity that can donate electron and act as primary and secondary metabolites, has the ability to reduce oxide intermediates (Yen, 1995). Copper reduction based on a complex mixture of antioxidant and appropriate selection of reaction time (Prior *et al.,* 2005). This drawback can be handled in different ways, adjusted to use a fixed reaction time and used strong complexing agent (EDTA) to stop the reaction within 3 minutes.

The standard L-ascorbic acid showed the highest reducing capacity. All the methanol, ethanol and chloroform extracts showed dose dependent reducing capacity. Among the three extracts of *Typhonium trilobatum* root of ethanol extract showed relatively better cupric reducing antioxidant capacity. Different concentration (200, 100, 50, 25, 12.5, 6.25 µg/ml) of root extracts of this plant were subjected to this investigation and the methanol, ethanol and chloroform extracts showed maximum absorbance showed at highest concentration whereas standard L-ascorbic acid showed the highest reducing capacity at the same concentration (Figure 4.4).

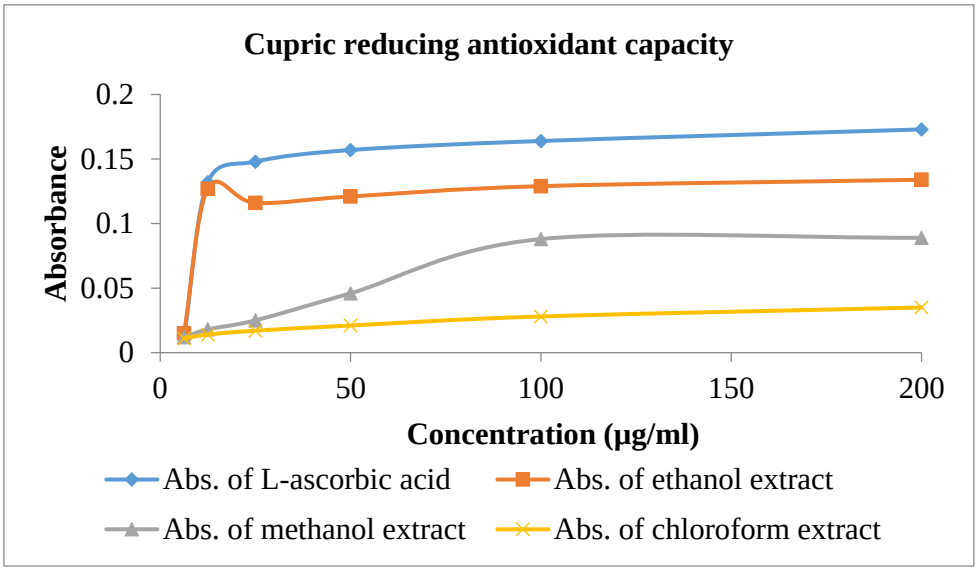

Figure 4.4: Cupric Reducing Antioxidant Capacity (CUPRAC) of root extracts *of Typhonium trilobatum*

4.4.1.3 Nitric Oxide Radical Scavenging Assay

Nitric oxide (NO) is generated from amino acid L-arginine by vascular endothelial cells, phagocytes and involved in the regulation of various biochemical reaction. Nitric oxide is classified as a free radical because of its unpaired electron and displays important reactivity with certain types of proteins and other free radicals. The toxicity of NO becomes adverse when it reacts with superoxide radical, forming a highly reactive peroxynitrite anion (ONOO−)

(Nagmoti *et al.*, 2011). Excess generation and accumulation of NO are implicated in cytotoxic effects observed in various disorders like AIDS, cancer, alzheimer's disease and Arthritis (Sainani *et al.*, 1997). Overproduction of NO can mediate toxic effects such as DNA fragmentation, cell damage and neuronal cell death.

The present study was based on the principle that sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions (Ebrahimzadeh *et al.*, 2010).

The nitrite radical scavenging assay was carried out on the methanol, ethanol and chloroform extracts of *Typhonium trilobatum* roots from a concentration of 200 to 6.25 µg/ml. Percentage free radical scavenging was plotted against the concentration of the extracts, as shown in Figure 4.5.

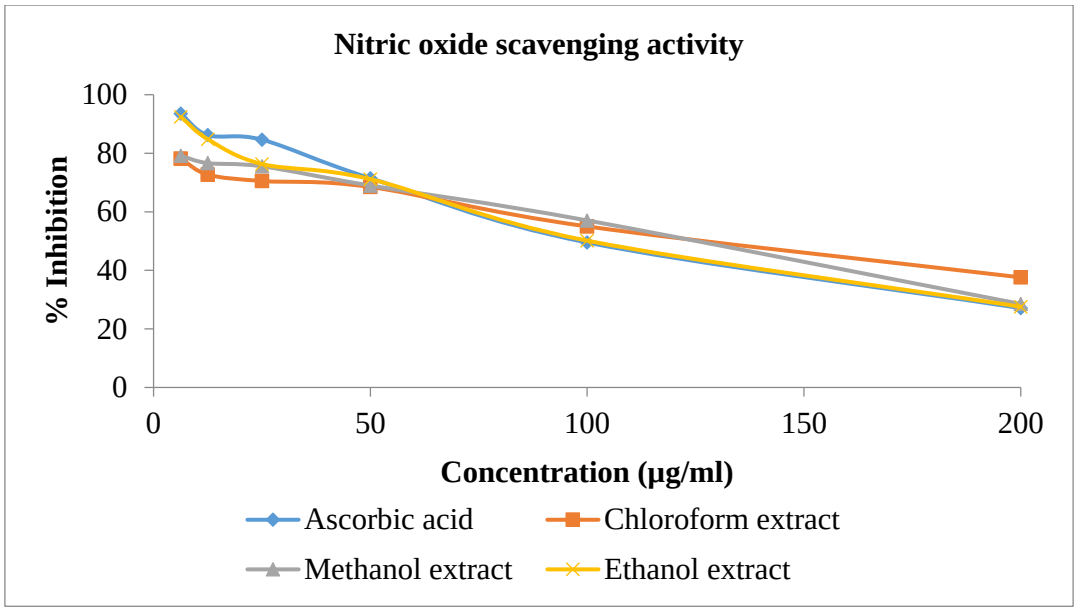

Figure 4.5: Percent of inhibition of nitric oxide based free radicals by root extracts

The root exhibited antioxidant activity through competing with oxygen to scavenge for the nitrate radical which was generated at physiological pH in an aqueous environment. The antioxidant activity depend on the concentration of the extracts. Increasing the concentration of the extracts did not result in an increase in the nitrite radical scavenging activity. Suppression of NO- release may partially be attributed to direct NO- scavenging, as all extracts decreased the amount of nitrite generated from the decrease of sodium nitroprusside *in vitro*. Different concentration (200, 100, 50, 25, 12.5, 6.25 µg/ml) of roots extracts of *Typhonium trilobatum* were subjected to investigate the nitric oxide scavenging activity and the methanol, ethanol and

chloroform extracts showed maximum activity of at low concentration whereas standard L-ascorbic acid showed the same. All the three extracts showed very good activity (Methanol extract showed 64.32% inhibition, ethanol extract showed 67.08% inhibition and chloroform extract showed 63.77% inhibition) that is even slightly lower than standard (68.76% inhibition). IC_{50} of ascorbic acid was found 119 µg/ml. In comparison to standard, methanol, ethanol and chloroform extracts showed IC_{50} values of 121, 117 and 133 µg/ml respectively (Table 4.5). The result revealed that ethanol extract of *Typhonium trilobatum* roots has maximum nitric oxide scavenging capacity.

Table 4.5: IC_{50} values of different extracts of *Typhonium trilobatum* roots in Nitric oxide scavenging assay

Sample	IC_{50} value (µg/ml)
L-ascorbic acid	119
Methanol extract	121
Ethanol extract	117
Chloroform extract	133

4.4.1.4 Scavenging of Hydrogen Peroxide

H_2O_2 was considered poorly reactive because of its weaker oxidizing and reducing capabilities. Biologically, it acts as a toxicant to the cell by converting it into hydroxyl radical in the presence of metal ions and superoxide anion and also produces singlet oxygen through reaction with superoxide anion or with Hypochlorous acid (HOCl) or chloramines in living system. Hydrogen peroxide can degrade certain heame proteins, such as hemoglobin, to release Fe ions (Lee *et al.,* 2004) and therefore the hydroxyl radical scavenging activity of phyto extracts was measured. In this study, scavenging of hydrogen peroxide of different extracts of *Typhonium trilobatum* roots is presented in figure (Figure 4.6). Among the three extracts, chloroform extract showed good potential antioxidant activity (67.29% inhibition) compared with standard L-ascorbic acid (73.41% inhibition). Methanol extract also showed antioxidant activity (54.55% inhibition) whereas ethanol extract showed 51.78% inhibition.

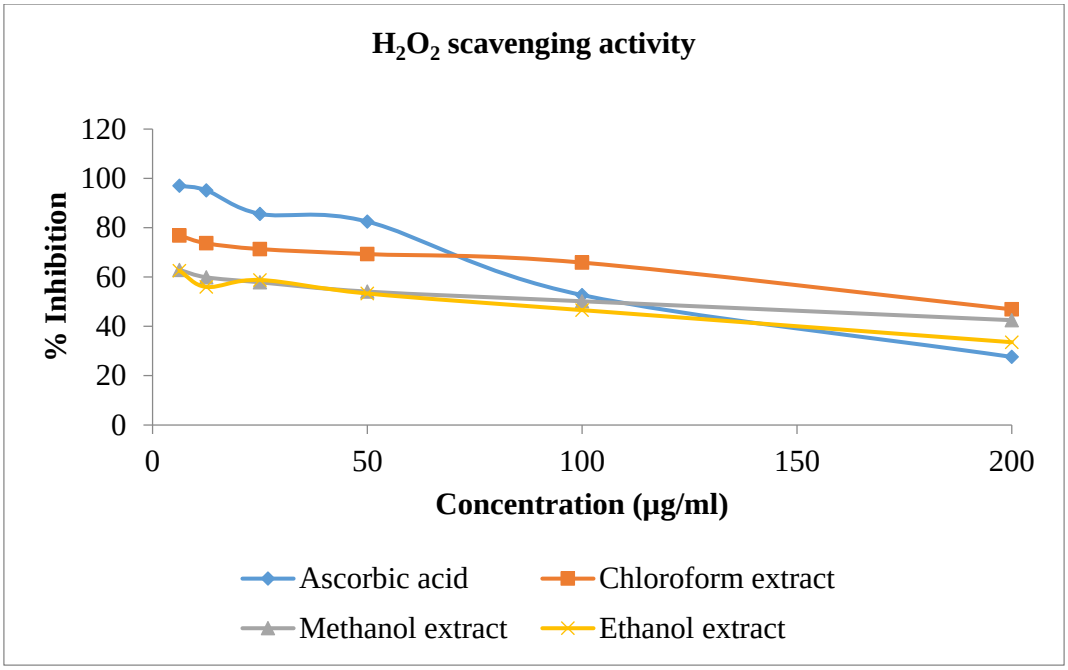

Figure 4.6: H_2O_2 scavenging activity

IC_{50} of ascorbic acid was found 128 µg/ml. In comparison to standard, methanol, ethanol and chloroform extracts showed IC_{50} values of 110, 78 and 184 µg/ml respectively (Table 4.6). The result revealed that methanol extract of *Typhonium trilobatum* roots has maximum H_2O_2 scavenging capacity and this capacity was found to be decreased in following order: Ascorbic acid> Chloroform extract> Methanol extract> Ethanol extract.

Table 4.6: IC_{50} values of different root extracts of *Typhonium trilobatum* in H_2O_2 scavenging assay

Sample	IC_{50} value (µg/ml)
L-ascorbic acid	128
Ethanol extract	78
Methanol extract	110
Chloroform extract	184

4.4.2 Thrombolytic Activity

Investigation of the thrombolytic activity of the *Typhonium trilobatum* root extracts were carried out using a simple and rapid *in vitro* clot lysis model. On the basis of the result obtained in the present study we can say that the *Typhonium trilobatum* root extracts have no thrombolytic activity compared with the standard. Streptokinase used as a standard showed 93.79% clot lysis (Figure 4.7) whereas methanol extract showed 13.01% which is very much

lower thrombolytic activity compared to standard. Ethanol and chloroform extracts showed lower clot analysis of 10.91% and 12.82% respectively.

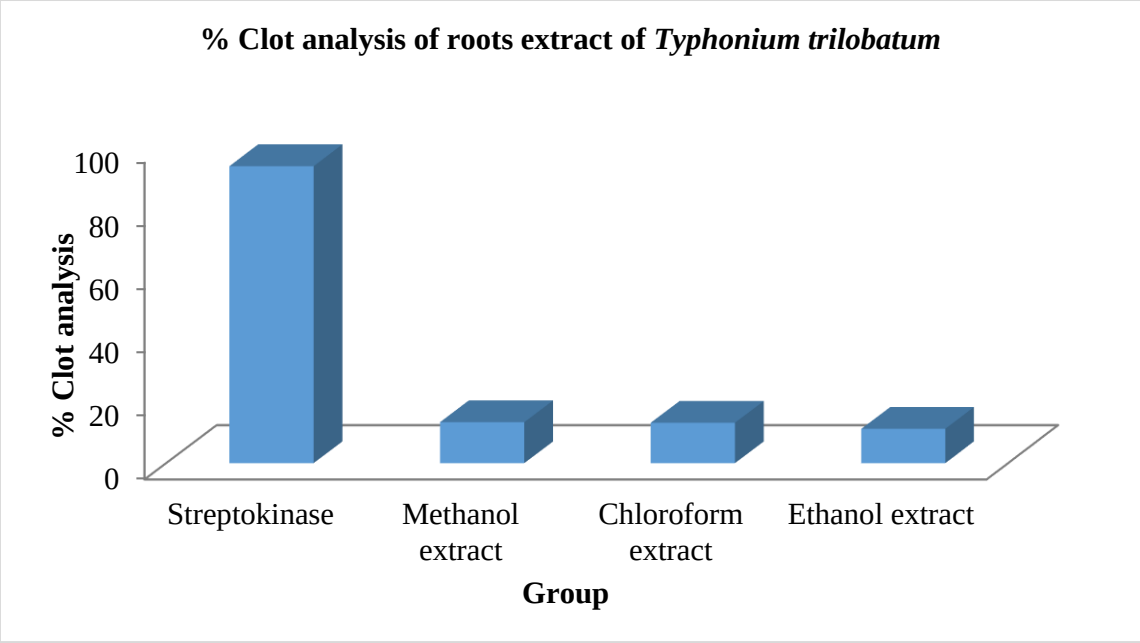

Figure 4.7: Percent of clot analysis of root extracts of *Typhonium trilobatum*

4.4.3 Membrane Stabilizing Activity

In hypotonic solution induced haemolysis, standard Acetylsalicylic acid (0.1 mg/ml) showed 64.87% inhibition of RBC haemolysis whereas the methanol extract, ethanol extract and chloroform extract, at a concentration of 1 mg/ml, showed 57.06%, 61.06% and 56.49% inhibition (Figure 4.8) of RBC haemolysis respectively. The results revealed that although all the root extracts have very good potential of membrane stabilizing activity, ethanol extract showed higher % of inhibition of haemolysis that are even much higher than the standard. Also, it was noted that all the extract showed dose dependent membrane stabilizing activity. The mode of action of the extract and standard anti-inflammatory drugs could be connected with binding to the erythrocyte membrane with the subsequent alternation of the surface changes of the cells. This might have prevented physical interaction with aggregation agents on promote dispersal by mutual repulsion of like charges which are involved in the haemolysis of red blood cell. It has been reported that certain flavonoids exerted profound stabilizing effect on lysosomal membrane both *in vivo* and *in vitro* while tannins and saponins possess ability cations there by stabilizing erythrocyte membrane and other biological macromolecules (Hess, 1972; Oyedapo *et al.*, 2004). It was noted that ethanol extract showed the highest membrane stabilities effect due to the presence of flavonoid. The lowest membrane stabilizing activities observed

with chloroform extract due to the presence of other phytochemical constituents which masking of the action of membrane stabilizing activities by other phytoconstituents. As per the results, it could be inferred that the extracts of *Typhonium trilobatum* capable of stabilizing bovine red blood cells membranes against hypotonic induced lyses. The plant therefore could be regarded as a natural source of membrane stabilizers and was capable of providing an alternative remedy for the management and treatment of inflammatory related disorders and diseases.

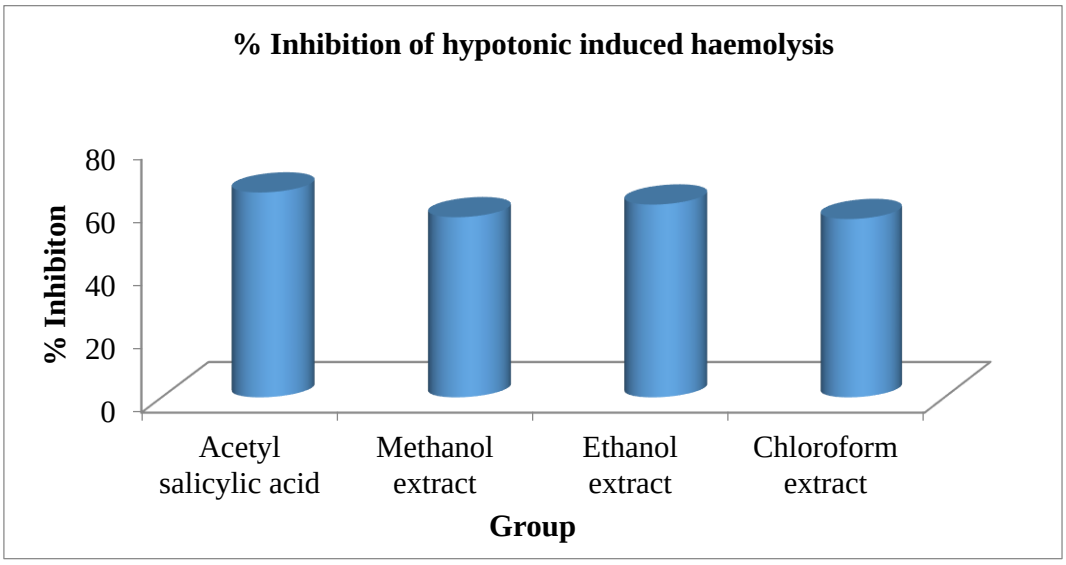

Figure 4.8: Percent inhibition of hypotonic induced haemolysis

In heat induced haemolysis, methanol extract, ethanol extract and chloroform extract showed 78.81%, 33.06% and 68.17% inhibition (Figure 4.9) of RBC haemolysis respectively whereas standard Acetylsalicylic acid showed 46.87% inhibition of RBC haemolysis. Methanol and chloroform extracts showed better potential in heat induced haemolysis that are even much higher than the standard. As lysosomal membrane stabilization contributes to protect cells from inflammation, the present investigation suggests that the membrane stabilizing activity of *Typhonium trilobatum* leaf extracts may play a very significant role in development of anti-inflammatory drugs.

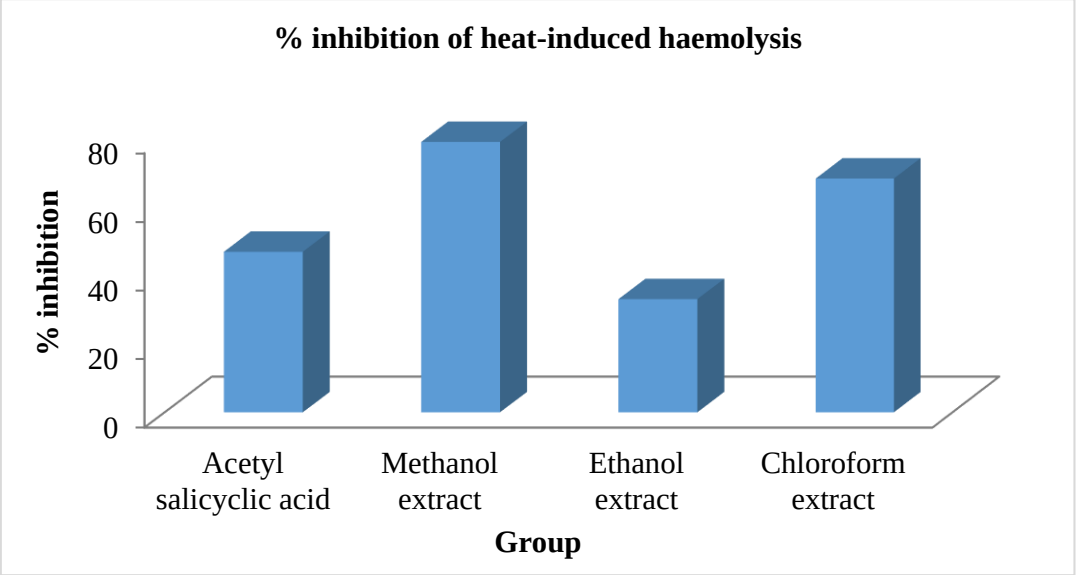

Figure 4.9: % inhibition of heat induced RBC haemolysis of *Typhonium trilobatum* roots.

4.5 *In vivo* Bioassays

4.5.1 Anti-depressant Activity (Forced Swimming Test)

FST is still one of the best models of antidepressant test. This is low–cost, fast, reliable model. The prevention and management of stress disorders remain a major clinical problem. Hence it is very important to address these problems and find effective remedies. Though several drugs are available, all are associated with some limitations and there is an urgent need for alternative medications for these disorders (Ratheesh *et al.*, 2007). In this work, it was demonstrated that the administration of 100 mg/kg doses of the each extract of *Typhonium trilobatum* in mice was able to induce antidepressant effects. In the forced swimming test, the extract can decrease the immobility time in rats with mild sedative effect. It was found that *Typhonium trilobatum* can produce antidepressant like activity at a dose of 100 mg/kg body weight in a dose dependent manner. The decrease in the immobility time is accompanied by the increase in swimming time.

An antidepressant drugs reduce the exploratory behavior depending upon the concentration. At present, the study revealed that all extract of methanol, chloroform and ethanol significantly reduces the number of head dips and the number of line crossings where the indicator of exploratory behavior. The findings from the present investigation indicate that *Typhonium trilobatum* possesses significant antidepressant activity as shown (Figure 4.10) by its mitigating effects on different experimentally induced stress models in mice.

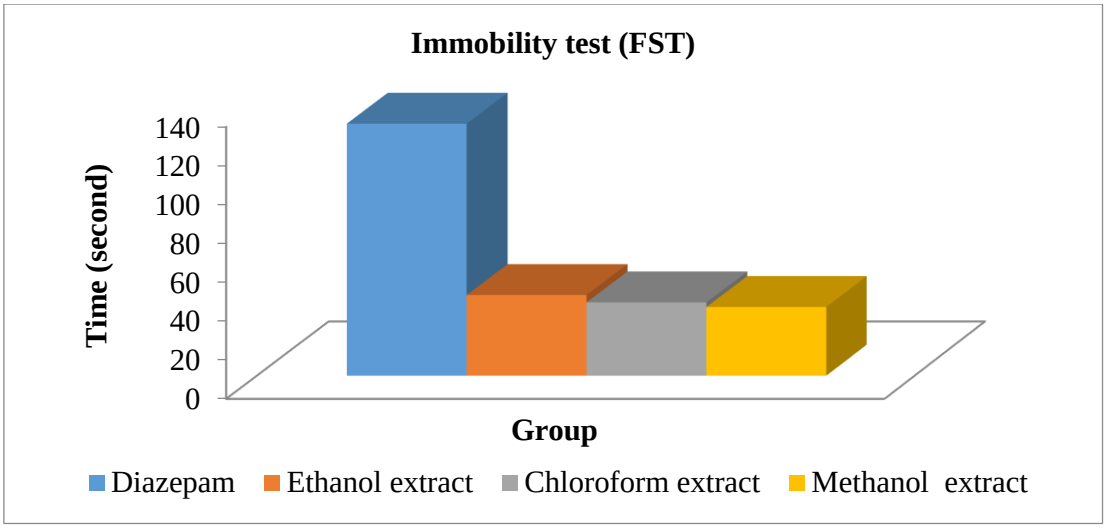

Figure 4.10: Immobile time in swimming test after administration of samples

4.5.2 Acute Toxicity

In this study, the mice in the treated groups were administrated with crude extract, respectively. The mice were monitored each hour until 12 hours for any toxic signs and mortality. The clinical symptom is one of the major important observations to indicate the toxicity effects on organs in the treated groups (Eaton, 1996). During the 6 hour and 12 hours of period acute toxicity evaluation (Table 4.7), mice which are orally administrated with each extract at single dose 100 mg/kg showed no overt signs of distress and there were no observable symptoms of neither toxicity nor deaths. All of the mice displayed no significant changes in behavior. Apart from the physical appearance features such as skin, fur and eyes were found to be normal, this indicates that the administration of the crude extract has negligible levels of toxicity.

Table 4.7: General appearance and behavioral observations for control and treated groups

Observation	6 Hour	12 Hour
Skin and fur	Normal	Normal
Eyes	Normal	Normal
Mucous membrane	Normal	Normal
Behavioral patterns	Normal	Normal
Salivation	Normal	Normal
Lethargy	Normal	Normal
Sleep	Normal	Normal
Coma	Normal	Normal
Diarrhea	Normal	Normal

Chapter 5: Conclusion

Root extracts of *Typhonium trilobatum* were subjected to phytochemical screening and *in vitro* and *in vivo* pharmacological evaluations to validate the traditional use and to find out any other therapeutic activity of the plant. Phytochemical screening revealed the presence of phenols, flavonoids and carbohydrate in root parts of the plant. *In vitro* antioxidant activity determined the potential of root extracts by assessing its scavenging capacity. Ascorbic acid, total phenolic and flavonoid contents were determine which has antioxidant activity. The root extracts has no thrombolytic activity but also have good membrane stabilizing capacity. After pharmacological studies with root extracts of *Typhonium trilobatum* has mild anti-depressant activity. The result clearly indicates that the extracts of *Typhonium trilobatum* may be a very important contributor in different drug discovery including antioxidant and anti-depressant drugs. The present study indicated a better chance of anti-tumor potential of the plant that might be revealed in near future. Therefore, further investigation on *Typhonium trilobatum* to isolate new bioactive compounds might be the next step to be followed.

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